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Effects of Adding α-tocopherol to Brahman Bull Chilled Semen on Sperm Quality, Lipid Peroxidation, Membrane Integrity, and DNA Integrity

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ABSTRACT

During storage at low temperatures, the spermatozoa quality changes due to cold shock and free radicals. Diluent supplementation with antioxidants is an effort to maintain the quality of spermatozoa during storage. This study aimed to evaluate the α-tocopherol effect in CEP extender on sperm quality, membrane integrity, and lipid peroxidation during storage at 4°C-5°C. This was a laboratory experiment that compared the use of 2 mM of α-tocopherol in CEP with no addition of α-tocopherol (as control) in five bulls. Semen was collected from Brahman bulls, diluted in CEP with and without α-tocopherol, and stored at 4°C-5°C. Sperm motility and viability were investigated by a light microscope at a ×400 magnification using Eosin-Nigrosin staining. Moreover, membrane integrity was evaluated by lipid peroxidation using the MDA assay and hypoosmotic swelling test. The sperm motility, viability, and membrane integrity were higher in CEP with α-tocopherol. Lipid peroxidation was significantly different between the treatment and control groups. The α-tocopherol supplementation in the diluent CEP could maintain the spermatozoa quality during storage at 4°C-5°C.

Keywords

α-tocopherol, Brahman bull, Cauda epididymal plasma, Frozen, Spermatozoa

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Abbreviations

AI: Artificial insemination
BSA: Bovine serum albumin
CEP: Cauda Epididymal Plasma
EDTA: Ethylenediaminetetraacetic acid
MDA: Malondialdehyde
PCR: Polymerase chain reaction
ROS: Reactive oxygen species
SNI: Indonesian National Standard
TBA: Thiobarbituric acid

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Introduction

Some procedure in sperm preservation (storage) is performed at 4°C-5°C and freezing conditions, which affects sperm quality [1, 2]. Therefore, fresh semen must comply with a standard quality to be frozen, such as 70%-80% of motility and 80%-90% of viability [3]. The storage of semen at a temperature of 4°C-5°C can maintain the quality of spermatozoa for up to 2-3 days [4]. Moreover, this method does not depend on the availability of liquid nitrogen as a preservation agent. However, by modifying the diluent media, the storage time can be extended. Several studies showed an extension of storage time by using INRA96 diluent which could maintain the quality of bovine spermatozoa for up to 4 days [5], in commercial diluent Megapor S.L. Spain was able to maintain the quality of boar spermatozoa for up to 10 days [6].

Spermatozoa preservation at low temperatures cannot prevent the presence of free radicals. It reduces the acrosome integrity, motility, plasmalemma function, and viability, and induces chromatin damage [7, 8]. The freezing of spermatozoa preservation has been also reported to decrease spermatozoa motility and viability. The spermatozoa head region surrounding the plasma membrane is most susceptible to gluttony. Spermatozoa heads have been reported to swell more than 50% [9]. Moreover, it triggers spermatozoa of RNA expression before and after freezing on its preservation [10]. Fresh semen commonly contains not only spermatozoa cells but also other cells, such as leukocytes and epithelial cells, before storage. These cells and immature spermatozoa are the sources of ROS [11].

Lipid peroxidation is caused by free radical and spermatozoa damage [12]. Lipids, proteins, and DNA oxidation are usually induced by high ROS levels in cells [2, 13]. There are high lipid membrane phospholipids in spermatozoa that lead to high ROS generation [14-16]. Spermatozoa with damaged and abnormal morphology produce higher ROS than spermatozoa with normal morphology [17]. Dead spermatozoa are the main source of ROS during storage at low temperatures [18, 19].

Antioxidants are required to reduce lipid peroxidation in the extender. Vitamin E (α-tocopherol) is one of the non-enzymatic antioxidants that protects polyunsaturated fatty acids, cell components, or cell membranes from oxidation by free radicals [20].

The addition of α-tocopherol in the semen diluent affects the spermatozoa quality at low-temperature conditions. It is able to maintain motility and protects spermatozoa from damage [21]. A study reported that the addition of α-tocopherol caused a significant change in the diluents of Madura bull spermatozoa [22]. Furthermore, α-tocopherol can maintain the integrity, motility, and viability of the spermatozoa membrane on the Simental bull after storage at 4°C-5°C. High-quality semen has a motility of ≥ 70% and a viability of ≥ 75%. The use of antioxidants in diluents is useful for maintaining spermatozoa quality [23].

The CEP diluent was first developed by Verberckmoes et al. [24] who used it to store bovine semen at a temperature of 4°C-5°C with the constituent components mimicking the physical and chemical conditions in the cauda epidydimal plasma of cattle [25]. They modified the concentration of egg yolk and replaced the antibiotic gentamicin with penicillin-streptomycin, and managed to maintain the quality of spermatozoa until day 8. The CEP diluent is also able to maintain the quality of sexed spermatozoa during storage at low temperatures [26]. This study aimed to evaluate the sperm quality, membrane integrity, and DNA integrity of Brahman bull that was stored in CEP with or without α-tocopherol at 4°C-5°C.

Results

As shown in Table 1, the mean percentage of sperm motility was not significantly different on days 0-3 between the control and treatment groups. However, a significant difference was found in spermatozoa motility in CEP with α-tocopherol compared to without α-tocopherol on days 4-7. Based on our study, sperm motility was better in CEP with α-tocopherol than without α-tocopherol. There were no significant differences ($p > 0.05$) in the viability percentage of sperms from day 0 to 4. However, on days 4-7, the percentage of sperm viability was higher ($p < 0.05$) in diluents with α-tocopherol than without α-tocopherol (Table 1).

Membrane Integrity

The motility and viability of spermatozoa were assessed along with spermatozoa function during storage based on their membrane integrity. The evaluations of membrane integrity percentage are illustrated in Table 2.

There was no significant difference in membrane integrity at the beginning of storage (days 0-4). On the other hand, there was a significant difference in membrane integrity in each treatment after day 5. The best results were in the CEP diluent using α-tocopherol on day 7.

Lipid Peroxidation

The MDA levels were measured to determine the existence of radicals inside and outside the cell. The MDA results can be seen in Table 3.

Table 1.
Effects of α-tocopherol addition to cauda epididymal plasma extender on sperm quality (Motility % and Viability %) during seven days (D) of storage at refrigerator temperature (4°C -5°C)

Treatments	% Motility in days							
	D0	D1	D2	D3	D4	D5	D6	D7
CEP without α-tocopherol	58.83^{a} ± 0.84	55.83 ^{a} ± 0.83	52.50 ^{a} ± 1.43	48.33 ^{a} ± 1.66	44.17 ^{b} ± 0.96	41.67 ^{a} ± 1.67	38.33 ^{b} ± 0.85	35.00 ^{b} ± 0.00
CEP + α-tocopherol	56.67^{a} ± 0.83	55.00^{a} ± 0.00	50.00 ^{ab} ± 0.00	50.00 ^{a} ± 0.00	49.17 ^{a} ± 0.83	46.67 ^{a} ± 1.66	45.83 ^{a} ± 0.83	45.00 ^{a} ± 0.00
	% Viability in days							
CEP without α-tocopherol	80.76^{a} ± 1.06	70.94 ^{ab} ± 0.76	73.00 ^{ab} ± 0.59	69.54 ^{a} ± 0.68	62.61 ^{ab} ± 1.53	49.46 b ± 2.68	47.17^{b} ± 1.78	45.50 ^{b} ± 1.00
CEP + α-tocopherol	79.2 ^{a} ± 0.20	71.45 ^{a} ± 0.82	69.77 ^{b} ± 0.77	68.37 ^{ab} ± 0.43	64.13 ^{a} ± 0.31	60.58 a ± 1.31	58.44 a ± 0.47	56.10 a ± 0.11

CEP: Cauda epididymal plasma
$^{a-b}$ uppercase letters in the same column indicate significant differences based on Student's T-test (α = 5%)

Table 2.
Effects of α-tocopherol addition to cauda epididymal plasma extender on membrane integrity % during seven days (D) of storage at refrigerator temperature(4°C-5°C)

Treatments	Membrane integrity (%)							
	D0	D1	D2	D3	D4	D5	D6	D7
CEP without α-tocopherol (control)	79.59 ^{a} ± 1.06	72.61^{a} ± 0.72	71.76 ^{a} ± 0.65	68.93 ^{ab} ± 0.35	60.86 ^{ab} ± 1.60	48.55 ^{b} ± 2.57	46.41 ^{b} ± 1.99	43.98 ^{b} ± 1.33
CEP + α-tocopherol	78.23 ^{a} ± 0.32	70.53 ^{a} ± 0.99	68.84 ^{ab} ± 0.95	67.69 ^{a} ± 0.35	65.92 ^{a} ± 0.63	65.66 ^{a} ± 1.94	60.91 ^{a} ± 1.02	56.29 ^{a} ± 0.33

CEP: Cauda epididymal plasma
$^{a-b}$ uppercase letters in the same column indicate significant differences based on Student's T-test (α = 5%)

Table 3.
Effects of α-tocopherol addition to cauda epididymal plasma extender on malondialdehyde values during four days (D) of storage at refrigerator temperature (4°C-5°C)

Treatments	MDA value			
	D1	D3	D5	D7
CEP without α-tocopherol (control)	0.222 ± 0.191 ^{a}	0.306 ± 0.179 ^{a}	0.390 ± 0.055 ^{a}	0.613 ± 0.234 ^{a}
CEP + α-tocopherol	0.116 ± 0.007 ^{b}	0.168 ± 0.168 ^{b}	0.175 ± 0.018 ^{b}	0.182 ± 0.008 ^{b}

CEP: Cauda epididymal plasma; MDA: malondialdehyde
a-b uppercase letters in the same column indicate significant differences based on Student's T-test (α=5%)

There were significant differences in MDA levels between treatments. A significant difference was found on days 1-7 of storage. The lowest MDA levels were shown in the α-tocopherol treatment, while the highest MDA levels were observed in the control group (CEP without α-tocopherol).

DNA Integrity

DNA integrity during storage may change due to the presence of free radicals. DNA integrity was assessed by sequencing a gene that controls spermatozoa motility (NAD1-1) (Table 4).

Table 4.
Data of NAD-1 sequences

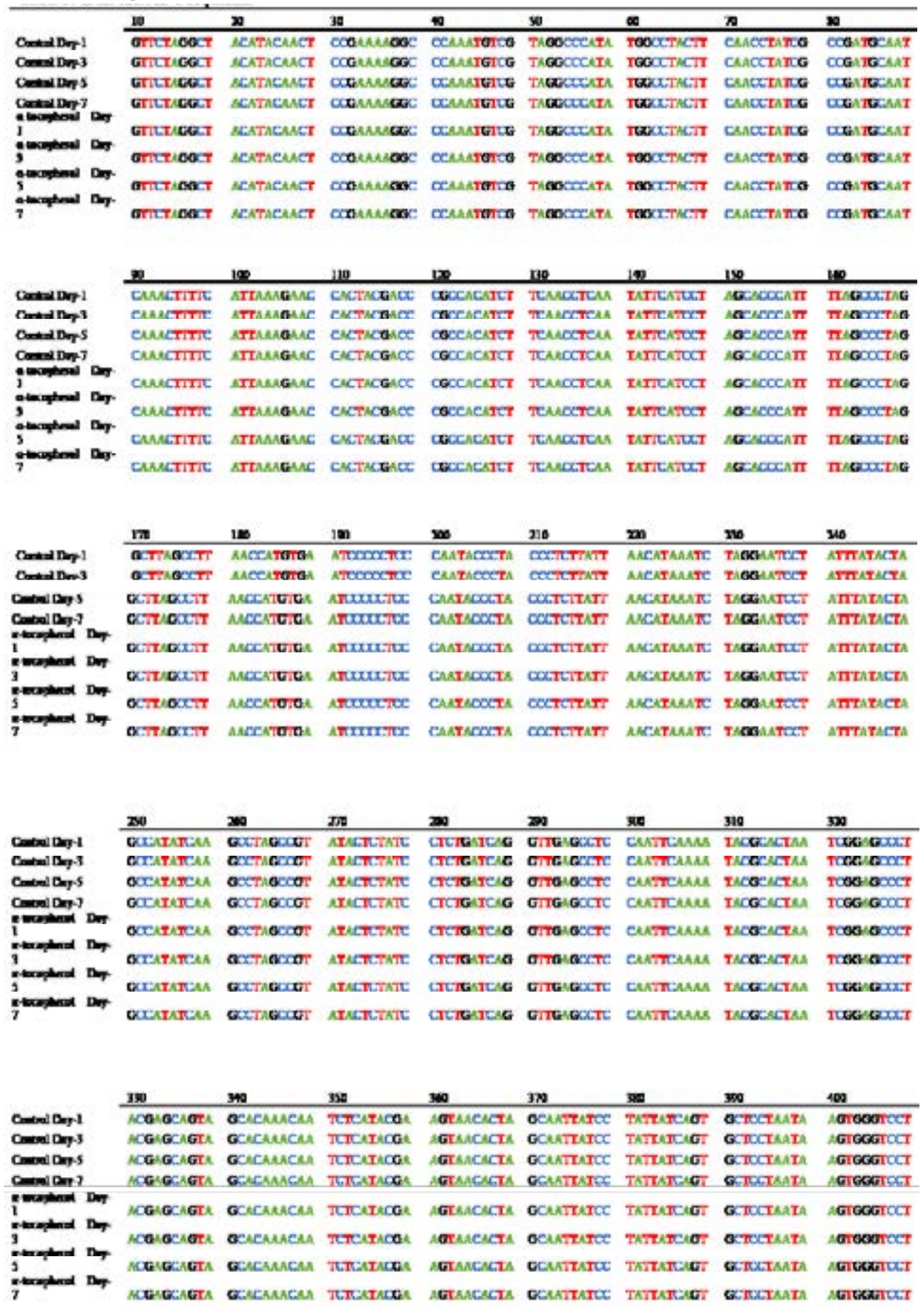
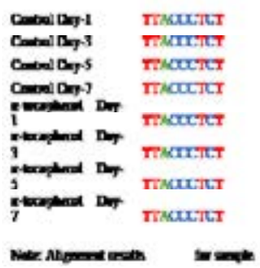


Table 4 - Cont'd



Discussion

At the beginning of storage, no significant differences were found between the control and treatment groups. It demonstrated that short storage time did not affect the spermatozoa quality. This result also indicated that the dilution process of semen in CEP with and without α-tocopherol was a success. Therefore, it did not cause a significant change in spermatozoa quality. It was also supported by the spermatozoa quality such as motility and viability become slowly.

In the present study, we revealed that the CEP media was very suitable for the storage of bull spermatozoa, although the semen was categorized as rejected semen. The CEP media could maintain the spermatozoa quality from the rejected semen during the storage process at 4°C-5°C. These findings are also in line with Verbeckmoes [24] and Ducha [25, 27, 28]. The CEP media provides chemical and physical conditions to store bulls' spermatozoa. The basic diluent of CEP was developed by imitating the physical and chemical conditions of bovine epididymal plasm which has an osmolarity in the range of 290-300 mosm, contains ionic components Na, K, Ca, Cl, Mg, P, bicarbonate, energy source molecules (i.e., fructose and sorbitol), molecules for pH balance (i.e., tris and citric acid), as well as antibiotics and egg yolk [24, 27]. Another research [24] showed that CEP was able to maintain the quality of bovine spermatozoa for up to 6 days at a temperature of 4°C-5°C with the addition of 20% egg yolk. Furthermore, the replacement of gentamicin with penicillin-streptomycin showed that CEP could maintain the quality of bovine spermatozoa for up to 8 days [25].

The addition of α-tocopherol to the CEP diluent strengthens the ability to maintain the quality of Brahman bull spermatozoa during storage at 4°C-5°C. It was similar to the SNI for AI until the seventh day of storing Brahman bull semen. The CEP media without α-tocopherol keeps the quality of bull spermatozoa from the rejected semen until the fifth day of storage. The increase in free radicals during the storage of spermatozoa at low temperatures may be due to the production of free radicals from dead or abnormal spermatozoa. The addition of antioxidants into the diluent media tends to minimize the production of free radicals [19].

Sperm stored at a low temperature can be damaged by free radicals. α-tocopherol is known as an antioxidant that reduces free radicals and can improve the quality of spermatozoa. We found that the addition of α-tocopherol into the CEP extender did not protect the spermatozoa of the Brahman bull at the beginning of storage. We observed no significant difference between the control and treatment groups. Furthermore, free radicals in the diluent can be overcome by antioxidants in spermatozoa cells during five days of storage. Therefore, the extent of damage to cells was low. α-tocopherol plays the role of an antioxidant and protects the cells or other compounds against free radicals by donating one hydrogen atom from the OH group to free radical compounds, resulting in tocopheroxyl radical compounds that are more stable and non-damaging. It can stop the propagation process when lipid peroxidation occurs in the membrane of spermatozoa [29]. The concentration of α-tocopherol can vary considering the percentage of lipids in the environment, affecting the solubility of vitamin E and the morphology of spermatozoa. Therefore, the type and composition of the diluent media and the type of animal will influence the dose of α-tocopherol [30].

There were significant differences in membrane integrity, motility, and viability on the sixth day of storage. These conditions were caused by free radicals in the diluent. Antioxidants were unable to reduce free radicals in the cell, especially in CEP diluents without adding α-tocopherol. The decrease in spermatozoa quality, such as motility, viability, and membrane integrity was faster in diluents without α-tocopherol compared to diluents containing α-tocopherol. Brahman bull spermatozoa stored in CEP diluents without α-tocopherol showed a low quality of sperm on the fifth day of storage. It was due to the presence of free radicals in semen. This finding is similar to buffalo sperm which has a higher quality with an extender containing α-tocopherol or vitamin C [31]. These results indicate that α-tocopherol provides the best protection for the plasma membrane of spermatozoa. α-tocopherol is known as an antioxidant that prevents lipid peroxidation during storage in bovine spermatozoa [32].

The integrity of spermatozoa DNA during storage was assessed based on the profile of the genes encoding spermatozoa motility (NAD1-1). Spermatozoa motility was a major parameter in determining spermatozoa quality at the center of AI. The motility parameter is determined by the ability of spermatozoa to fertil-

ize eggs. These results revealed no changes in genes during various treatments until day seven of storage. It indicated that the presence of free radicals during storage for up to seven days did not damage the cell and DNA structure. Gene sequences did not show any changes in either the control or treatment groups, before or after storage. The process of storing the liquid at 4°C-5°C was simpler than the freezing method and fewer free radicals existed in the liquid method than in the frozen technique. The freezing process can trigger damage to the integrity of the spermatozoa membrane, compared to storage at 4°C-5°C as happened in the freezing process of human spermatozoa. Freezing storage triggers damage in chromatin integrity by oxidative stress [33]. The storage of human spermatozoa at freezing temperatures damages DNA integrity [34]. DNA integrity did not damage after storage at 4°C-5°C, while DNA integrity was damaged after freezing [35].

Assessment of both sperm motility and sperm viability by light microscope was an important limitation of the present study. However, computer-aided sperm analysis technology (CASA) is more accurate for such assessments. Moreover, other concentrations of α-tocopherol or combinations of other antioxidants with α-tocopherol may be required to obtain better results on bull spermatozoa stored at 4°C-5°C. Therefore, further research is needed in this important field of research concerning spermatozoa preservations. It is necessary to study the effect of adding α-tocopherol to the CEP diluent in which each diluent has compositional characteristics, especially lipids that can affect α-tocopherol solubility. Consequently, it can influence the effectiveness of α-tocopherol in counteracting free radicals.

Conclusion

The sperm motility, viability, and membrane integrity were higher in CEP with the supplementation of α-tocopherol. Our present study showed that lipid peroxidation was significantly different between the treatment and control groups. The CEP with α-tocopherol supplementation could maintain the spermatozoa quality during storage at 4°C-5°C.

Materials & Methods

Preparation of Extender with α-tocopherol Addition

The CEP extender in the present study was prepared according to Verbeckmoes et al. [18, 27] with antibiotic, egg concentration, and a different method (water jacket method) [20]. The following compounds were included in the CEP extender: BSA 2.0 gr/l, CaCl2(H2O)2 3.0 mmol/l, citrate acid mmol/l, fructose 55 mmol/l, KH2PO4 20.0 mmol/l, KCl 7.0 mmol/l, NaH2PO4 8.0 mmol/l, NaHCO3 11.9 mmol/l, NaCl 15 mmol/l, MgCl2(H2O)6 3.0 mmol/l, penicillin 1000 IU, streptomycin 1 gr, sorbitol 1.0 gr/l, and tris 133.7 mmol/l. Next, 2 mM of α-tocopherol (Sigma, USA) was added to the CEP extender while the control group was not supplemented with α-tocopherol.

Bull Semen Collection and Preparation

Fresh semen of the bull was obtained from AI Center in Singosari, Malang, Indonesia by an artificial vagina. Bulls' semen was obtained twice a week to gain optimum semen quality. The fresh semen was observed to evaluate motility and viability before being diluted. The low quality of semen, according to the SNI, was continued for the process of dilution and freezing. If the quality was below the SNI, semen was rejected. According to the SNI, low-quality semen does not meet the requirements of the freezing process. For example, the motility of spermatozoa is less than 70% and the viability percentage is less than 75%, and it is called rejected semen. Fresh semen was diluted 25 times in a CEP extender with 2 mM α-tocopherol and without antioxidants as the control group. Spermatozoa were stored at refrigerator temperature (4°C-5°C) in darkness conditions at 25×106/ml concentration.

In the present study, we used fresh semen with low quality. We evaluated the level of spermatozoa degradation from fresh low-quality semen (rejected for the freezing process) during storage at low temperatures on CEP diluents with α-tocopherol as an antioxidant, compared to those without α-tocopherol.

Sperm Motility

Spermatozoa motility was measured by observing semen using a light microscope to determine the percentage of progressive motility. Spermatozoa were taken by a stick glass on days 0 and 8 in the CEP-2 extender with and without egg yolk. Next, it was placed on an object glass and covered with cover glass. The observation was conducted under a light microscope with ×200 magnification. Evaluation of the motility-based method of Garner and Hafez [36] was performed by two people to determine sperm motility.

Sperm Viability

Sperm viability was assessed using Eosin-Nigrosin staining to gain the permanent slides. Nigrosin provides a dark background to recognize viable cells. Non-viable sperms had red or dark pink heads and viable sperms had white or faintly-pink heads.

Membrane Integrity

Membrane integrity was determined using the hypoosmotic swelling test. It was performed by incubating 100 µl semen in the control and treatment groups with 1 ml of 125 mOsm/l hypoosmotic (0.31 g sodium citrate and 0.565 g fructose in 50 ml H2O) at 37°C for 30 min. Afterwards, 0.2 ml of the mixture was spread using a coverslip on a warm slide after incubation. The observation was performed using 200 sperms under light microscopy at a magnification of ×400. Sperms with swollen or coiled tails were recorded [37].

Lipid Peroxidation

Sperm oxidative levels were determined using the MDA assay-based TBA reaction. A total of 1 ml in each spermatozoa treatment (five technical replications) was incubated in 0.7 ml of tris buffer (100 mM, pH 7.4), 10 mM of iron sulfate, and 100 mM sodium ascorbate at 37°C for 60 min. This reaction was stopped by adding 1 ml of 10% trichloroacetic acid on ice for 15 min. Samples were immediately centrifuged at 7800 rpm for 15 min and the supernatant was removed. A total of 2 ml supernatant was added with 1 ml of 1% TBA into endogenous peroxidation. Next, the specimen was boiled for 10 min and kept in a cool condition. The absorbance levels were read by spectrometry at 530 nm [38, 39].

DNA Integrity

DNA extraction

DNA extraction was conducted according to the method of Silva et al. [40]. Each semen treatment was divided into three aliquots. DNA was extracted with phenol-chloroform according to Hanson and Ballantyne [41]. A total of 100 µl of semen aliquots were centrifuged at 6000 rpm for 5 min. Pellet was resuspended using 1 ml Tris EDTA solution (1 mM EDTA, 100 mM NaCl, 100 mM Tris-HCl, pH 8.0) and centrifuged at 6000 rpm for 5 min.

A volume of 500 µl of lysis buffer containing 25 mM EDTA, 0.5% sodium dodecyl sulfate, 100 mM NaCl, and 10 mM Tris-HCl with a pH of 8.0 was added to the pellet, followed by 22 µl of 0.1 M dithiothreitol and 25 µl proteinase K (QIAGEN GmbH). Then, the sample was incubated at 55°C for 3 h, with a vortex in each hour. A total of 500 µl phenol was added, balanced with Tris at pH 7.8, and followed by a vortex. Centrifugation was conducted at 10000 rpm for 3 min. The supernatant was transferred to another tube, along with 300 µl phenol and 300 µl chloroform, followed by vortex and centrifugation at 10000 rpm for 3 min. The supernatant was placed into a new tube and 700 µl chloroform was added. The mixture was centrifuged and gently vortexed again. The supernatant was placed into a new tube containing 95% ethanol. Samples were incubated at -20°C for 4 h. Each sample was centrifuged at 10000 rpm for 10 min, and the supernatant was removed. Then, the DNA pellet was dried and resuspended with 50 µl 1X TE buffer (100 mM Tris-HCl, pH 7.5, 0.25 M EDTA), and stored at -20°C until further analysis.

DNA concentration and purity

Genomic DNA concentration and purity were assessed by measuring optical density in a Genesys Spectrophotometer. DNA absorbance was read at 260 nm to determine DNA concentration. DNA purity was evaluated by determining the A260/280 ratio and comparing it with a reference value of 1.8 [42].

DNA visualization on agarose gel

The existence and quality of DNA genomes were analyzed with a 0.8% agarose gel. Five microliters of sperm pellets were stained with 0.3 µl of Blue-Green (LGC Biotecnologia, Cotia, SP, Brazil), and electrophoresis was performed on the agarose gel. The results were visualized under an ultraviolet transilluminator (Vilber Lourmat, Paris, France), and the image was digitalized (C7070; Olympus, Tokyo, Japan).

DNA amplification

Genomic DNA amplification with PCR used ND1 gene primers. The ND1 gene is 925 bp and the PE Biosystems ABI 3700 DNA Analyzer 96 capillary electrophoresis system could not accommodate the 925 bp sequence.

Table 5 presents the primers for segments 1 and 2 of the ND1 sequence.

The first segment of the ND1 sequence used the antisense primer (3'–5'), and the second segment used the sense primer (5'–3') to align the two segments. Primers were selected specifically for mtDNA to distinguish between mitochondrial and nucleic DNA.

Data Analysis

Data including average motility, viability, and membrane integrity were analyzed by analysis of variance to determine whether the treatment caused an effect. Moreover, the Student's T-test (α = 5%) was utilized to assess the differences between treatments.

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Authors' Contributions

ND: Conceptualization, Supervision, Formal analysis, Writing original draft; DH : Writing original draft, Review and Editing. Data Curation; WB : Methodolgy, Formal Analysis, Review and Editing; TS : Data Curation, Formal Analysis, Writing Original Draft; AA : Formal Analysis, Writing-Review and Editing, Methodology; SW : Methodology, Data Curation, Writing-Review and Editing.

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Conflict of interest

The authors declared no conflict of interest for this research.

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Table 5.
Primers in the mtDNA sequencing procedure

Segment		Sense/antisense primer	Primer sequence
1	457 bp (3131–3588)	BOVNADH1S (sense)	ATTCCCATCCTATTGGCC
		BOVNADH1A(antisense)	GAGAGGGTAAAGGACCCACT
2	488 bp (3567–4055)	BOVNADH1S (sense)	TAAGTGGGTCCTTTACCCTC
		BOVNADH1A (antisense)	ATGTTTGTGGTGGGATGC

species in liquid and frozen-thawed bufallo semen. Anim Reprod Sci. 2009;114(1-3):125-134. Doi: 10.1016/j.anireprosci.2008.10.002.

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